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Determination of Human Tumor Necrosis Factor α by a Highly Sensitive Enzyme Immunoassay

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Tumor necrosis factor α (TNF-α) is a polypeptide produced primarily by monocytes and macrophages. It is involved in a wide variety of immune reactions. A simple and sensitive microplate enzyme-linked immunosorbent assay for the detection of hTNF-α in serum, plasma, and cell culture supernatants is described. The method is based on the use of horseradish peroxidase in biotin–streptavidin amplification system which is performed in Nunc StarWell. This system has enabled us to achieve a sensitivity of 0.1 pg hTNF-α/ml of the sample. The assay is calibrated to the World Health Organization (WHO) standard for hTNF-α (87/650). The within-run coefficient of variation ranged from 3.7 to 5.9 and the between-run coefficient of variation ranged from 8.0 to 9.9. The results obtained by the proposed method and by a commercially available kit (DRG hTNF-α ELISA) correlated well (*n* = 20, *r* = 0.956).

Key Words: **human TNF-α; enzyme immunoassay; streptavidin–biotin.**

Tumor necrosis factor α (TNF-α) is an extremely potent peptide cytokine which serves as an endogenous mediator of inflammatory, immune and host defense functions. Several substances originally described for their biological activities have been identified as TNF-α, cachectin, macrophage cytotoxin (MCT), hemorrhagic factor, macrophage cytotoxic factor (MCF), and differentiation inducing factor (DIF).

Abbreviations used: BSA, bovine serum albumin; DIF, differentiation including factor; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; hTNF-α, human tumor necrosis factor α; IL-1, interleukin-1; MCF, macrophage cytotoxic factor; MCT, macrophage cytotoxin; PBS, phosphate-buffered saline; TMB, tetramethylbenzidine; WHO, World Health Organization.

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It is generally thought that TNF-α is not produced constitutively by normal cells, but needs to be induced potently by invasive stimuli in the setting of both endoplastic and infectious disease (1–4). In this respect, macrophages and monocytes are thought to be the cells which contribute most to the local and systemic TNF-α response to bacterial, viral and parasitic organisms and products.

Bioassays for the quantification of TNF-α, including the cytotoxic assay on murine fibroblasts have been used for several years. However, TNF-α and IL-1 have several common biological properties which influence the results. Therefore, these properties should be taken into account in the bioassay development method. The cytotoxic assay is unaffected by IL-1. However, it is time-consuming and might be susceptible to interference by other substances (5–7).

Various enzyme immunoassays, with different sensitivities are available for detection of minutes amounts of many analytes (8–10). One of the most important amplified enzyme immunoassay is biotin–streptavidin amplification system (11). Based on the principle of this system, our group developed an ELISA method for a faster and more sensitive quantification of hTNF-α in body fluids as well as in cell culture media, in comparison to the commercially available kits. To increase surface/volume ratio, Nunc C8 StarWell with eight inside fins has been used. In this case, the incubation time can be reduced by a factor equal to the square of the surface/volume increase factor.

MATERIALS AND METHODS

Natural and recombinant human tumor necrosis factor α (TNF-α) was purchased from Sigma Chemical Co. (Sigma-Aldrich Chemie GmbH, Germany). Two different kinds of anti- hTNF-α antibodies and other chemicals were also obtained from Sigma Co.

Coating of solid phase with anti-hTNF-α. To increase the surface/volume ratio of the test environment, the NUNC-immuno module C8 StarWell was used as solid phase for fixing the anti-hTNF-α. The

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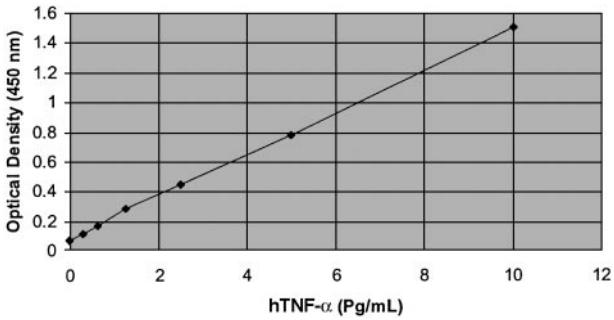

FIG. 1. The calibration curve for the recombinant hTNF-α.

coating antibody was diluted by coating buffer (0.1 M carbonate/bicarbonate, pH 9.6), by a ratio of 1:100 (1 μg protein/well). One hundred microliters of diluted anti-hTNF-α was added to each well and incubated overnight at room temperature. The plates were then washed five times with washing buffer (0.2 M PBS, pH 6.8). Then 200 μl of the blocking buffer was added to each well. The blocking buffer is made of 0.1 M phosphate buffer (pH 6.8) plus BSA (0.5%).

The wells were incubated at room temperature for 1 h. The plates were then washed five times with the second washing buffer (PBS plus 0.05% Tween 20).

Biotinylation of anti-hTNF-α. Biotin was conjugated to anti-hTNF-α by the modified method of Guesdon, Ternynck, and Avramease (12–14) as follows: one hundred microliters of the biotin *N*-hydroxy-succinimide solution in DMSO (3 mg/ml) was added to 2 ml of anti-hTNF-α in 0.2 M $NaHCO_3$/KCl (15 mg/ml) and incubated at room temperature for 10 min. Then 400 μl of NH_4Cl was added and the mixture was kept for 15 min at room temperature. The mixture was then dialyzed against PBS at 4°C for 24 h.

The optimal concentrations of the biotinylated antibody for the assay were established using, as diluent, Tris–HCl buffer (10 mM, pH 8.5) containing 10 mg BSA/milliliter.

Conjugation of HRP with streptavidin. Conjugation of HRP was achieved using the Nakane and Kawaoi method (15, 16). The diluting buffer of the biotinylation step has been used for the titration and dilution of the HRP streptavidin conjugate.

Standards. A stock solution (500 pg/ml) of the recombinant hTNF-α was prepared in 50 mM phosphate buffer (pH 7.2) containing BSA(5 mg/ml) and NaCl (150 mM). Serial dilutions containing 10, 5, 2.5, 1.25, 0.63, and 0.31 pg/ml of hTNF-α were prepared using the same phosphate buffer. The standard solution needs to be prepared freshly.

HRP substrate. The substrate solution should be prepared just before use. The temperature of the substrate solution should be in the range of 20–25°C in order to obtain optimal reproducible results.

For each plate, 12 ml of the substrate buffer (0.11 M acetate buffer, pH 5.5) was mixed with 200 μl of the stock solution of TMB (6 mg/ml of DMSO) and 12 μl of H_2O_2 stock solution (3%). Meanwhile, 100 μl of the H_2SO_4 solution (1.8 M) is used as the stop reagent.

Assay procedure. Before the assay, all reagents and standard solutions are equilibrated to room temperature. Pipette 100 μl of the standards or the samples into the appropriate wells. Cover the plate and incubate it for 60 min at room temperature on a plate shaker (700 rpm). The wells need to be washed five times with the wash buffer (PBS with 0.05% Tween 20). Then 100 μl of the biotinylated antibody was pipetted into each well and incubated at room temperature for 60 min while shaking on a plate shaker (700 rpm).

The wells were washed five times with the washing buffer, following which the HRP conjugate (100 μl) was added to each and the plate was incubated for 30 min at room temperature while shaking (700 rpm) on the plate shaker.

TABLE 1

Intra- and Interassay Variability (Mean ± SD)

	Sample	*n*	Mean (pg/ml)	SD	CV%
Intraassay	A	10	8.3	0.3655	4.3
	B	10	2.3	0.1288	5.6
	C	10	0.41	0.0319	7.8
Interassay	A	10	8.7	0.4524	5.2
	B	10	2.6	0.1976	7.6
	C	10	0.46	0.0446	9.7

After the incubation time was over, the plate was washed five times with the washing buffer. The HRP substrate solution was then added to each well (100 μl per well). The plate was incubated at room temperature for 30 min on the shaker. The process was stopped by adding 100 μl of the stop solution to each well. The optical density of each well was measured at 450 nm.

RESULTS

Calibration Graph and the Precision of the Assay

Figure 1 shows the standard curve obtained using the standard recombinant hTNF-α with various concentrations ranging from 0.31 up to 10 pg/ml of the test samples. The detection limit based on the calibration graph is about 0.1 or 0.01 pg/well. The standards were calibrated to the WHO reference 87/650. One pg/ml of WHO standard corresponds to 0.04 WHO unit. The assay precision are shown in Table 1 and recovery data are presented in Table 2.

Specificity of the Assay

The amounts of hTNF-α in various standard solutions were determined in the presence of IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, and TNF-β. No interference and cross-reactivity was detected in presence of 10 ng/ml of above cytokines. The results of specificity of the assay are presented in Table 3.

Human Serum/Plasma TNF-α

This method may be used to measure hTNF-α in serum, plasma and cell culture supernatants. Serum

TABLE 2

Recovery Results (pg/mL)

Sample TNF-α	Added	Calculated	Observed	Recovery %
10	2.3	12.3	11.9	96.7
5	2.3	7.3	7.0	95.8
2.5	2.3	4.8	4.6	95.8
1.25	2.3	3.5	3.6	101.4
0.62	2.3	2.9	3.1	105.8
0.31	2.3	2.6	2.8	107.2

Note. A known quantity of the hTNF-α has been added to each patient's sample.

TABLE 3

Specificity Results of the Assay

Cytokine (10 ng/mL)	% Cross-reaction	Cytokine (10 ng/mL)	% Cross-reaction
Recombinant hTNF-α	100	TNF-β	<0.05
Natural hTNF-α	100	IL-2	<0.01
IL-1	<0.01	IL-3	<0.01
IL-4	<0.01	IL-6	<0.01
IL-7	<0.01	IL-8	<0.01

must be removed as soon as possible from the clot and keep at 4°C. Plasma can be collected on sterile EDTA or heparin tubes and rapidly separated after centrifugation. The normal values of hTNF-α in sera and plasma of twenty two healthy laboratory personnel were established to be lower than 7.9 and 4.8 pg/ml, respectively. Testing of RPMI with different lots and concentrations of fetal bovine serum has shown that the developed ELISA method is not disturbed by culture media. Figure 2 shows the normal values of hTNF-α in sera and plasmas.

Method Comparison

The data presented in Fig. 3 show the performance of our method compared to commercially available hTNF-α ELISA kit from DRG Company. The TNF-α levels of 20 samples were determined by two methods. The values obtained had correlation coefficients of 0.956. The DRG kit showed positive bias relative to our method.

Sensitivity

The limit of detection, defined as the least amount of hTNF-α significantly different from zero standard at 95% confidence limit (i.e., mean for zero $\pm$ 2 SD), was 0.1 pg/ml.

DISCUSSION

To examine the effect(s) of some anti neoplastic natural products on the release of TNF-α, a highly sensitive method of measurement of this cytokine in the cell culture media and human serum or plasma, was needed. The reported bioassays methods of hTNF-α measurements lack sensitivity and specificity, besides of being time consuming in comparison to immunoassay techniques. On the other hand, the sensitivities of the present reported hTNF-α enzyme immunoassay methods are in the range of 1 pg/ml (17–21). To enhance the sensitivity of the assay, we developed an enzyme immunosorbent assay with biotin

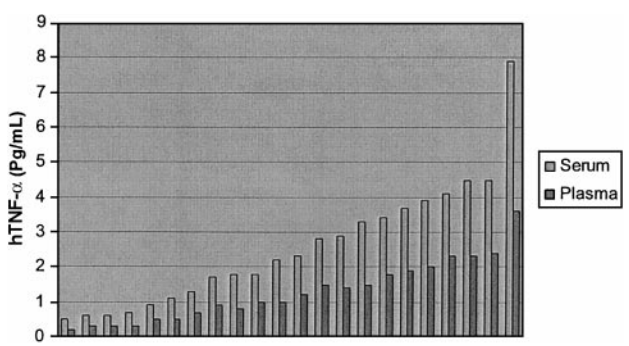

FIG. 2. The levels of hTNF-α in sera and plasmas of 22 laboratory personnel.

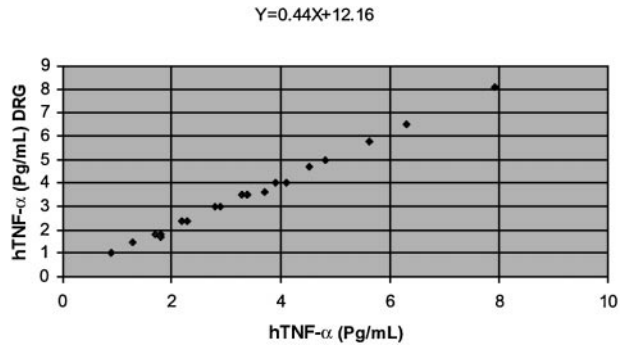

FIG. 3. Correlation between hTNF-α concentration measured by our method and by DRG ELISA kit.

Streptavidin Amplification System

The assay has a lower detection limit of 0.1 pg/ml (95% confidence interval) and it is highly reproducible (intra- and interassay coefficients of variations were between 4.3 to 7.8 and 5.2 to 9.7, respectively). On the other hand, the assay time has been reduced by replacing the flat-bottom wells with C8 starwells which are equipped with eight inside fins. This replacement resulted in an enhancement of the liquid covered surface area by a factor around 1.5 compared to the standard flat bottom wells. The level of hTNF-α in 22 healthy laboratory personnel was found to be lower than 10 pg/ml, by the developed biotin–streptavidin kit and the commercially available DRG ELISA kit. In addition our data indicated that the level of serum hTNF-α is higher than the plasma level. Since sampling conditions can affect the measurements, strict precautions must be taken during sampling to avoid blood cell stimulation which will falsely increase the hTNF-α levels. There was good agreement between the hTNF-α biotin–streptavidin ELISA results and those obtained by the DRG ELISA kit ($n = 20$, $r = 0.956$). The assay recognizes both natural and recombinant hTNF-α. The capability of the assay in detecting free TNF-α or the receptor-bound hTNF-α had to be worked out. The influence of various drugs, aberrant sera and rheumatoid factor has not been investigated thoroughly enough.

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